ANNUAL PROGRESS REPORT

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PRINCIPAL INVESTIGATOR: Thomas F. Anderson

Associate:	Dr. Catherine F. Rappaport	(Full time to May 31, 1953)
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	Mr. Robert L. Tyson	(Medical Student from Columbia University; full time during summer only; not on contract)
	Mr. Harrison MacMichael	(Medical Student, University of Penna.; full time during summer only; not on contract)
	Mr. James Ray	(Graduate Student in Physics; not on contract)
	Mr. Chester Baran	(Full time since October 1953; student in College Collateral Course)

TITLE OF PROJECT: Physical, Chemical, and Biophysical Characterization of Viruses and Virus Systems.

Objectives:

A. To determine the nature and functional anatomy of virus particles.

B. To elucidate the mechanisms of infection of host cells by viruses and of specific reduplication of virus elements.

C. To promote naval applications of the knowledge gained, especially in the fields of medicine and physics.

ABSTRACT OF RESULTS:

A. Since Start of Project:

1) Cofactors for Virus Adsorption.

We have discovered and studied extensively an inherited requirement of phages T4 and T6 for aromatic L-amino acids like tryptophan which must be taken up by these phage particles before they can be adsorbed on host cells.

2) Electron Microscopy.

We have developed a method of preparing specimens for the electron microscope which eliminates gross artifacts due to surface tension forces in removing the ambient liquid. The method involves raising the temperature above the critical point of the ambient liquid (so its surface tension vanishes) and then allowing the ambient fluid (now in the gaseous state) to escape. When the three-dimensional structures of specimens are thus preserved it is seen that:

a) heads of phage particles T2, T4, and T6 and the unrelated phages T1 and T5 are shaped like hexagonal bi-pyramids with membranes 125 A thick, surrounding an internal structure (desoxyribosenucleic acid, DNA);

b) phage particles having tails (T2, T4, T6, T5) are adsorbed to host cells by the ends of their tails;

c) immature intracellular particles of T2 have the typical polyhedral structure of empty T2 heads with no tails attached and membranes which are 250 A rather than 125 A thick;

d) from observations of flagellae and from theoretical considerations of their thermal vibrations it is concluded

that the tensile strengths of dried flagellae exceed some 3000 gm/cm^2.

3) Intracellular Growth of Phage T3.

Growth of T3 involves the multiplication of one or more non-infectious units. For the first half of the latent period no infectious particles (not even the infecting one) can be recovered from the cell. During the second half of the latent period the non-infectious units produce infectious daughter particles which can be liberated by sonic disruption of the cell before the end of the latent period when it would normally lyse.

4) Sonic Reactivation of Antiserum-Neutralized T3.

T3 which has been neutralized by antiserum can be reactivated by sonic vibration which presumably strips antibody from the particle.

5) Effects of Crystal Violet.

Crystal violet (4×10^{-7} gm/ml) is lethal to complexes formed between T2 and host cells when the cells have been grown 24 hrs. on ammonium lactate medium before infection, but has no detectible effect on complexes which T2 forms with cells grown on Difco nutrient broth.

6) Osmotic Shock of Bacteriophages.

It was discovered that preparations of the large phages T2, T4, and T6 in concentrated solutions of NaCl or some other solute remain infectious if they are diluted slowly before they are assayed. However, if they are diluted rapidly, most of the infectivity is destroyed and most of the particles are seen to have lost the internal structure in their heads (which Herriott has shown to be DNA).

The fluorescence of the dye acriflavine is rapidly quenched when it combines with the DNA of T6 particles which have been heat-killed or osmotically shocked. It combines very slowly with the DNA of intact phage below 60 C but more rapidly in the presence of detergents. The combination is rapidly reversed by the addition of ions such as H^+, Na^+, or Mg^{++}, which presumably displace the dye from sites on the nucleic acid.

The fraction of particles in a given stock which resists "osmotic shock" depends on many factors including:

a) the nature of the solute,

b) the magnitude and rate of the drop in osmotic pressure,

c) the temperature, and

d) the previous history of the preparation.

Resistance to shock is thus a physiological rather than a genetic property of a phage particle. In the case of T6r in 2.5 M NaCl the equilibrium proportion of resistant forms increases as the temperature is raised. The rate of approach to equilibrium is very rapid at 45 C but is so slow at OC that by rapidly chilling a preparation from a high temperature, the particles can be kept in the resistant form for hours at OC even though at equilibrium less than 1% of them would be resistant to osmotic shock at this temperature.

B. <u>During Current Report Period:</u>

1) <u>Effect of NaCl Concentration on the Shockability of T6r.</u>

Using the above rapid chilling technique to "freeze" T6r particles in their physiological state, it was possible to determine the effect of salt concentration on the equilibrium between shockable and resistant forms. For example, if T6r is incubated

in water at 26 C, then diluted in 2.5 M NaCl at OC and tested, almost all the particles are found to be resistant; but if they are incubated in 1 M NaCl at 26 C, then diluted in 2.5 M NaCl at OC and tested, only 5% are found to be resistant. It is not yet known whether these effects are due to reversible changes in the head membranes of the particles or to changes in the state of the DNA inside their heads.

2) Two Factors Affecting the Stability of T6.

a) Instability in 0.035 M NaCl.

In the course of the studies just mentioned it was found in many experiments that diluted preparations of T6 are very unstable in a narrow range of concentrations of NaCl. The instability is maximal at 0.035 M, while in 0.01 M or 0.05 M NaCl the virus is stable. In other experiments and at higher virus concentrations no instability was observed suggesting that traces of organic material may stabilize the virus. The nature of this instability has not been determined, although it has not been observed in NaCl containing 10^{-5} M $CaCl_2$. In the presence of chelating agents like ethylene-diamine tetraacetic acid (versene) T6 is unstable over a broader range of NaCl concentrations than mentioned above.

b) Toxicity of H_2S and Cu^{++}.

Mr. Tyson confirmed an observation made last spring that H_2S solutions inactivate T6 preparations. Cupric ion at 10^{-6} M, with H_2S is much more toxic to T6 than is H_2S alone, while 10^{-6} M Cu^{++} alone is not toxic. Zn^{++} or the

chelating agent, "versene", failed to reverse the inactivation of T6 by H_2S.

3) Osmotic Shock of T4 during Adsorption on its Host.

a) Single Infection.

Mr. Tyson and Mr. Mc Michael both confirmed an early observation that the survival of a phage particle to osmotic shock increases shortly after the particle becomes adsorbed on a susceptible host cell in F medium. Working on the hypothesis that the particle would become resistant after completing its injection of DNA into the host, Mr. Tyson concentrated his attention on the conditions in the medium which are necessary for this phenomenon to occur. He found that injection absolutely requires substrate and in the presence of substrate is inhibited by 10^{-2} M KCN. The rate of injection measured in this way depends on the state of the host cells. It is fast in ammonium lactate medium and slow in nutrient broth.

b) Multiple Infection.

Mr. McMichael began experiments to see whether survival of newly-formed complexes to osmotic shock might not be higher if many virus particles are adsorbed than if only one is adsorbed per cell. The contrary was found to be the case: the presence of many virus particles on a cell reduces the complex's chance of survival to osmotic shock.

4) Spectrophotometry.

The sensitivity of T6 to H_2S and the effects of KCN or the

absence of a substrate suggested that the energy-producing cytochrome systems may be involved at the earliest stages of bacteriophage action on host cells. It also seems clear that the energy-producing systems are involved later in phage action, too, for the respiration of cultures being lysed by T4 drops to very low values indeed (Cohen and Anderson) whereas the respiration of mechanically broken cells is almost as high as that of whole cells. It would seem to follow that phage lysis inactivates either the dehydrogenase systems or the cytochrome systems through which they are coupled to molecular oxygen.

Accordingly, we have initiated a study of the spectral, properties of phage, host cells (E. coli strain B), and their interactions using the sensitive spectrophotometric methods developed in this laboratory by Dr. Britton Chance, and his collaborators, who have greatly assisted us in this phase of work.

a) <u>The Cytochrome System of E. coli B.</u>

It is found that both whole cells and cells broken in a fly press (which are treated with desoxyribonuclease to reduce viscosity) contain at least three of the cytochrome pigments: a_1, a_2, and b_1 as determined from difference spectra between oxidized and reduced preparations. In addition, an unidentified peak appears in difference spectra of whole cells at 506 mμ.

b) <u>Behavior of DPNH and Pyruvate in Whole Cells and in Broken Cells during Lactate Metabolism.</u>

Upon going anaerobic, respiring whole cells exhibit a sudden increase in DPNH absorption at 340 mμ which then slowly decreases with time. However, no change in

DPNH absorption is observed when broken cells become anaerobic, even though broken cells respire almost as rapidly as whole cells. Instead, as lactate is oxidized by broken cells, a progressive increase in absorption in the region of 370 to 310 mμ is seen. The absorption of the latter material agrees closely with that of pyruvate ion, is destroyed by KCN (as that of pyruvate would be), and furthermore, quantitatively agrees with the hypothesis that broken cells carry the oxidation of lactic acid only as far as pyruvic acid. Additional experiments show that neither whole cells nor broken cells metabolize pyruvate at pH7, the former presumably because pyruvate fails to penetrate the cytoplasmic membrane, and the latter presumably because some enzyme system or cofactor for pyruvate metabolism has been lost. Separate experiments showed that in air the broken cells rapidly destroyed absorption due to added DPNH. It may well be that the amplitude of the transient appearance of DPNH in broken cells is blocked by the pyruvate which accumulates in these preparations, shifting the equilibrium DPNH concentration to a very low value. In line with this idea we have observed that added pyruvate inhibits the respiration of press-broken B in lactate.

c) Light Scattering by Respiring and Anaerobic Cells in Ammonium Lactate.

One further observation may be worthy of mention here. When anaerobic 18 hour cultures of B are aerated in an

ammonium lactate medium, the light scattering increases steadily until the moment when the culture becomes anaerobic. At this point the scattering decreases to its original value at a logarithmic rate. Presumably the aerobic increase in scattering is due to the dynamic accumulation of material by the cell, which material is then slowly lost when the oxygen supply and hence a source of energy is exhausted.

5) Utilization of Glucose by Yeast Cells.

Dr. Rappaport has concluded her study of some factors regulating the utilization of glucose in baker's yeast. Since glucose can be used as the only source of both carbon and energy for the growth of many micro-organisms, factors regulating its utilization may be expected to regulate growth.

It was found that the metabolism of glucose varies with the glucose concentration. Respiration (oxygen uptake) is saturated at a concentration lower than that required to saturate assimilation (polysaccharide synthesis) which is, in turn, saturated before the different fermentations.

The increase in assimilation of glucose after respiration was maximal suggested that the rate of DPN linked oxidations, intermediary in glucose degradation, could be a determining factor in the regulation of assimilation. Dr. Chance had already observed spectrophotometrically that the steady-state level of DPNH increased after the respiratory system was saturated. This suggested the possibility that assimilation could be excluded during the metabolism of glucose by agents which decrease the steady-state level of DPNH.

Dr. Chance has shown that the steady-state level of DPNH may be decreased by 1) agents, such as acetaldehyde, which use DPNH by an enzyme not saturated during the utilization of glucose, and 2) by acids which can penetrate into the cell and suppress the DPNH forming systems.

It was found that acetaldehyde and a variety of acids did, indeed, exclude assimilation without inhibiting the rate of respiration. The effective concentrations of the acids, salicylic, benzoic, formic, proprionic, acetic and barbituric varied in a regular manner with their acid strength. Some amino acids were also effective, but with the exception of serine, the effect was delayed and variable. This could be due to the variable permeability of the cells to these ampholytes.

The metabolism of glucose was found to vary not only with glucose concentration but also with time. After prolonged utilization of glucose, and without any change in the rate of respiration, there was a sudden increase in the rate of one of the fermentation mechanisms and a decrease in the rate of assimilation. The metabolism at this time is similar to that induced by acids. Whether the sudden change is due to an accumulation of acids per se within the cell, or to other factors affecting the steady-state concentration of DPNH cannot be said without further study.

Growth studies of yeast on glucose indicated that growth varies with glucose concentration and with time in the same way as the assimilation in the resting cells. Of particular interest was the absence of growth below certain critical con-

centrations of glucose, and a partial inhibition of growth during metabolism at high glucose concentrations. The acids which excluded assimilation in the resting cells inhibited growth.

These results suggest that assimilation may be dependent on a critical steady-state level of DPNH. Factors affecting this level may be expected to change the total synthetic capacity of the cell. The fact that some of these factors arise during the normal course of metabolism may be important in the regulation of growth.

PLANS FOR FUTURE:

Immediate:

We plan to continue our studies on the roles of pigments like the cytochromes and DPNH in virus action and synthesis. This will involve comparing the behavior of normal cells with virus-infected cells and that of mechanically broken cells with cells which have been lysed by virus action. We shall also continue using osmotic shock of the viruses as a tool in studying the processes of infection.

Long Range:

As we see it, our basic task is to learn the physics and chemistry involved in the specific reduplication of biological units. At present the bacterial viruses seem to be the best material for this study, but we shall be on the lookout for material which is even more favorable for the pursuit of this basic task.

REPORTS AND PUBLICATIONS (DURING CURRENT REPORT PERIOD):

Anderson, T. F., "Stereoscopic Electron Microscopy of Phage Particles and of their Interaction with Host Cells." Riassunti delle Comunicazioni VI Congresso Internazionale di Microbiologia, Roma, 2,

183 (1953).

Anderson, T. F., "Stereoskopische Untersuchungen von Bakteriophagen mit dem Elektronenmikroskop." Physikalische Verhandlungen, Deutsche Gesellschaft f. Elektronenmikroskopie.

Anderson, T. F. and Carl F. Oster, Jr., "The Critical Point Method for Drying Electron Microscope Specimens" J. Appl. Phys. 24, 1416 (1953).

Anderson, T. F., "The Morphology and Osmotic Properties of Bacteriophage Systems," Cold Spring Harbor Symp. Quant. Biol., 18, (1953).